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Molecular Characterization of Virulent Newcastle Disease Virus Isolates from Chickens during the 1998 NDV Outbreak in Kazakhstan

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Abstract. Newcastle disease virus (NDV) infects domesticated and wild birds throughout the world and has the possibility to cause outbreaks in chicken flocks in future. To assess the evolutionary characteristics of 10 NDV strains isolated from chickens in Kazakhstan during 1998 we investigated the phylogenetic relationships among these viruses and viruses described previously. For genotyping, fusion (F) gene phylogenetic analysis (nucleotide number 47-421) was performed using sequences of Kazakhstanian isolates as compared to sequences of selected NDV strains from GenBank. Phylogenetic analysis showed that all newly characterized strains belonged to the genetic group designated as VIIb. All strains possessed a virulent fusion cleavage site (RRQRR/F) belonging to velogenic or mesogenic pathotypes with intracerebral pathogenicity indexes (ICPI) varying from 1.05 to 1.87.

Key words: chicken, fusion gene, Newcastle disease virus, phylogenetic characterization, virulent

Introduction

Newcastle disease virus (NDV) infects domesticated and wild birds throughout the world. The nature of the infection varies greatly with the species infected and NDV isolates vary from highly virulent to avirulent. The infection could result in symptoms of mild subclinical respiratory tract or enteric disease, often with neurological signs. More virulent strains cause either acute diarrhoea or dyspnoea and up to 90% of the birds die with hemorrhagic enteritis or tracheitis [1–3].

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Avian paramyxovirus type 1 (APMV1) or NDV is classified in the Paramyxoviridae family and possesses an approximately 15,000-nucleotide-long, negative-sense single-stranded RNA genome which contains six genes in the order of 3′-NP-P-M-F-HN-L-5′, encoding eight proteins the nucleoprotein, phosphoprotein, V, X, matrix, fusion, hemagglutinin–neuraminidase, and large polymerase proteins, respectively [2]. An NDV virus causes clinical effects that range from inapparent to severe, depending on a number of viral and host factors [1,2,4]. The primary molecular determinant for NDV pathogenicity is the fusion protein cleavage site [1] and the ability of specific cellular proteases to cleave the fusion protein of different

pathotypes [2]. Studies comparing the deduced amino acid sequence of the F_0 precursor of NDV strains varying in virulence for chickens showed that viruses highly pathogenic for chickens possess the amino acid sequence ^{112}R/K-R-Q-K/R-R-F^{117} in cleavage site whereas viruses with low pathogenicity possess the amino acid sequence ^{112}G/E-K/R-Q-G/E-R-L^{117} in the same region [1,2].

NDV was first isolated from chickens in Kazakhstan during 1980 [5] and it has been regarded as an endemic disease in some regions of the country since that time. In the succeeding years, an intensive vaccination program against NDV has been practiced in both large-scale poultry operations and small poultry farming. However, epizootic infections of velogenic NDV in chicken flocks have been more frequently reported since the middle 1990s [6]. In the study presented herein molecular characterization of virulent NDV strains isolated from chickens in several regions of Kazakhstan during 1998 were completed to determine the epidemiological relationships among these viruses and phylogenetic relationships to other worldwide NDV outbreaks.

Materials and Methods

Viruses

Ten NDV isolates were recovered from chickens on poultry farms in south-east and central regions of Kazakhstan during the 1998 NDV outbreaks. Mortality during the outbreak varied from 77% in young chickens and 17% in adult hens. The peak of mortality occurred in November of 1998, but increased mortality was also evident in January, June, and December. The clinical signs of disease were similar at all poultry farms. In early stages of disease there was depression and decreased egg production in layers. This was followed by evidence of diarrhea, pale combs, cyanosis, difficult breathing, paralysis, and swelling of the head. Necropsy findings included subcutaneous edema and hemorrhage of the head, tracheal edema with hemorrhage or necrosis, and segmental necrohemorrhagic enteritis. The observed clinical signs and high mortality were evidence of the severity of this Newcastle disease outbreak (NDV). NDV isolates were recovered from samples taken from ill or dead birds. Initial isolation of the virus was performed in 9–10 days old embryonated chicken eggs (ECE). Type of the viruses was determined in standard haemagglutination inhibition and neurarmnidase inhibition tests using specific antisera to the reference strains of parainfluenza and influenza virus. Allantoic fluids were harvested from ECE inoculated with the viruses and used as a stock for sequence analysis.

Biological and Molecular Characterization

Chicken embryo mean death time (MDT) induced by a minimal lethal virus dose was determined by standard procedures in 10-day-old chicken embryos inoculated with serial $\log_{10}$ dilutions of each isolate. MDT was estimated according to OIE recommendations [4] and isolates with MDT up to 60 h were characterized as velogenic, from 61 to 90 h as mesogenic, and more than 90 h as lentogenic.

The intracerebral pathogenicity index (ICPI) of NDV isolates was determined by intracerebral inoculation of 1-day-old chickens with 50 µl of infective allantoic fluid diluted 1:10 followed by daily observation during eight days post-inoculation. The ICPI was calculated according to OIE recommendations [1,4]. NDV isolates with an ICPI below 0.7 were considered to be of low virulence and those with an ICPI equal or greater than 0.7 were considered to be virulent.

RNA Isolation, Reverse Transcription, Polymerase Chain Reaction

RNA was extracted from infective allantoic fluid with an RNeasy Mini Kit (QIAGENE, GmbH, Germany) to obtain total RNA preparations. The RNA isolation procedure was done according to the kit's manual.

Primers used:

1.	CGCAGTGACCGCTGACCACGAG	1045F	22-mer
2.	ACAATCTTGCGCTCAA TGTCACTATT	614F	26-mer
3.	TAACTTGACTATGATTGACC CTGTCTG	601R	27-mer
4.	TTTGTGGCCCGAATACTGTAGTCA	1082R	24-mer
5.	ATCAGAATGCTGCCAACATCCTCC	480F	24-mer
6.	TGAGCCTCAAAGTTATCCC	1356R	19-mer
7.	GGAATATCAAGCGCCATGT	3232R	19-mer
8.	CACCCAACGTGCTGTCGCAGTGAC	F1005M	24-mer

Primer pairs were selected on the basis of alignment of different strains of NDV (Fig. 1).

Reverse transcription was completed using M-MLV (Promega, USA) in 5 μl of reaction mixture (2.7 μl sample, 0.84 μl water, 1 μl 5×-buffer for reverse transcriptase (Promega, USA), 0.19 μl 2 mM mixture of dNTPs, 0.25 μl 20 OE primer ***5′-ACAATCTTGCGCTCAATGTCAC-3′*** and 0.125 μl M-MLV) at 37°C during 60 min.

Polymerase chain reaction (PCR) was completed in a 25 μl reaction mixture (1 μl DNA matrix, 1 μl Taq-polymerase, 1 μl 20 OE direct and reverse primers, 2.5 μl 2 mM mixture of dNTPs, 2.5 μl 10X-buffer for PCR (Promega, USA) for 30 cycles in the "Eppendorf" thermocycler at next temperature/time: 94°C – 25 s, 55°C – 25 s, 72°C – 90 s. PCR was completed with primers 614F and 1356R. In case of low concentration of RNA/cDNA detection and absence of PCR fragment, second round of PCR with primers 1005F and 601R was completed (conditions: 94°C – 25 s, 53°C – 25 s, 72°C – 60 s 35 cycles). If detection was positive two sets of second round PCR were carried out with primers 614F/1082R and 1005F/1356R (conditions: 94°C – 25 s, 53°C – 25 s, 72°C – 90 s 35 cycles).

Prepared transcripts were analyzed by electrophoresis in 0.8% agarose gel. PCR products were purified by electrophoresis in 1% agarose gel. A slice of agarose containing the DNA of interest is "melted" by incubation in a solution containing chaotropic salt (e.g. sodium iodide) at a pH of 7.5 or less. Glass powder or silica gel is then added and the suspension is mixed to allow adsorption of DNA. The particles were recovered from the original liquid and washed by centrifugation and resuspended in high-salt-ethanol buffer. Finally, the pellet was resuspended in a solution with low or no salt at basic pH, the free particles were pelleted by another centrifugation, and the DNA-containing supernatant recovered.

Sequencing and Phylogenetic Analysis

Sequence analysis of PCR products was completed using a fmol DNA Sequencing System (Promega, USA) kit and the set of primers used for polymerase chain reaction (PCR). PCR products prepared after 25 cycles were analyzed by electrophoresis in 6% polyacrylamide gel (length 55 cm) containing 7 M urea on a "Macrophor" system at 1500 V.

Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted using the MegAlign program (Windows 32, MegAlign 5.00) in the Lasergene package (DNASTAR Inc. Madison, WI 53715, USA).

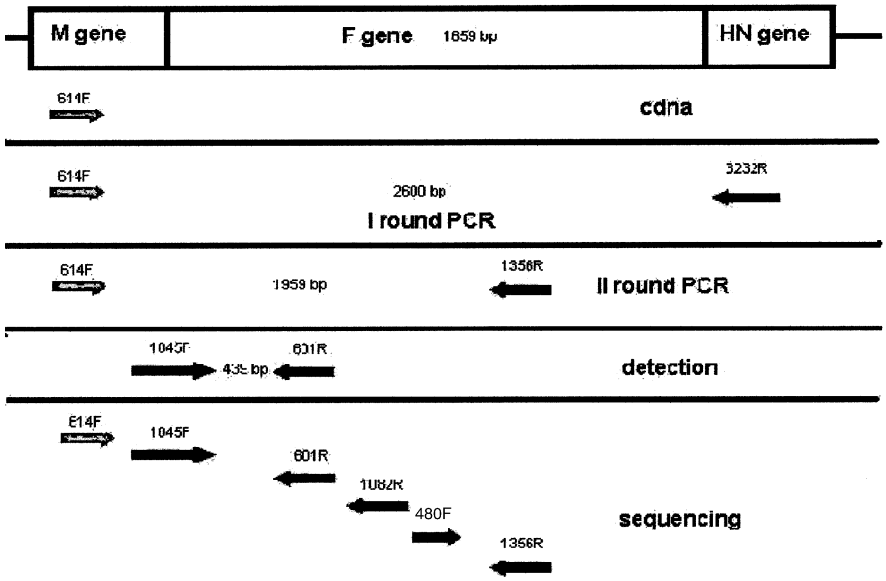

Fig. 1. Genomic position of primer annealing.

Phylogenetic trees were constructed for comparison of the nucleotide sequences of F gene fragments (from 47 to 421) by aligning with the ClustalW program and phylogenetic relationships by UPGMA. In addition to the 10 NDV strains isolated in Kazakhstan, 65 previously described NDV sequences representing different NDV genotype groups were used for comparison (Table 1).

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study are available in the GenBank under accession numbers AY847352–AY847361.

Results

Estimation of NDV Isolates Pathotype

The biological properties of NDV strains isolated in Kazakhstan during 1998 are presented in Table 2. Eight of ten NDV isolates were characterized as velogenic based on their MDT, which ranged from 54.2 to 62.4 h and ICPI that ranged from 1.58 to 1.87. Isolates KAZ-26-98 and KAZ-39-98 with MDT of 85.6 h and 70.4 h and ICPI of 1.05 and 1.42, respectively, were characterized as mesogenic. The deduced amino acid sequence of F protein cleavage site for all of these strains was determined to be ^{112}RRQRR$^{116}{}_{*}$ F^{117} and was characteristic of virulent NDV strains [2,4].

Comparison of Amino Acid Sequences in Variable Region of NDV F Protein

Deduced amino acid sequences of NDV F-gene fragments (residues 1–124) were aligned and the results are summarized in Table 3. Comparison of the F protein variable region (residues 1–124) of F-gene fragments had specific amino acid substitutions characteristic only for NDV strains isolated in Kazakhstan. The Kazakhstan isolates were most similar to genotype VIIb, but differ from other type VII viruses by having differing amino acid residues at positions 9, 19, 28, 101, 112, and 115 of the protein. The results of amino acid sequence analysis of Kazakhstan NDV isolates resulted in distinguishing differences of these strains from other worldwide

Table 1. List of representative NDV strains from GenBank database utilized for phylogenetic analysis

NDV strains	Genotype or subgroup	Accession number	Reference number
JP-Ishii-62	I	AB070385	18
BOR 74	I	NDVY16049	15
BOR 82	I	NDVY16176	15
Komarov-45	II	AY170137	11
BG-13-69	II	AF402107	10
HU-35-79	II	AF402102	10
Bea-45	II	NDVFPF	15
B1	II	NC_002617	15
Las-46	II	AF077761	15
Muktesvak	III	AY117022	11
H-Ph-02	III	AY170136	11
JP-Sato-30	III	AB070382	18
Grozn-47	IV	ND1243387	12
Simf-64	IV	NDV19017	12
Pok-70	IV	ND1243388	12
NY70181	V	AF001105	17
CA1085-71	V	AF001106	17
YU(VO)-C-91	V	AY117003	20
YU(SR)- 11-92	V	AY117006	20
Lebanon70	VI	AF001110	17
JP-Ibaraki-85	VI	AB070399	18
Astr-74	VI	NDI243391	18
JP-Niigata-85	VII	AB070403	18
JP-Niigata-89	VII	AB070410	18
ZA-5-68	VIII	AF136762	14
ZA-34-94	VIII	AF136773	14
IT-147-94	VIII	AF218132	13
IT-675-94	VIII	AY135744	13
China-GIF3	VII	AY390299	16
China-B1F4	VII	AY390297	16
China-HB	VII	AY390300	16
China-HEBC3	VII	AY390301	16
China-YMF3	VII	AY390313	16
China-HG97	VII	AY390302	16
China-YLF3	VII	AY390312	16
China-T02	VII	AY390307	16
TW-1-98	VII	AF083963	21
JP-Wakayama-85	VII	AB070405	18
JP-Hyogo-85	VII	AB070404	18
JP-Chiba-85	VII	AB070402	18
CZ-3898-96	VII	AF109883	9
CZ-677-96	VII	AY135743	7
CH-62-96	VII	AF109880	9
B-19-95	VII	AF001123	17
B-92-81	VII	AY135751	7
DE-143-95	VII	AF109881	9
D-85-96	VII	AF001119	17
E-1-93	VII	AF001126	17
D-16-93	VII	AF001113	17
D-83-95	VII	AF001118	17
NL-2-93	VII	AF001125	17
NL-1-93	VII	AF001124	17
RI-3-88	VII	AF001135	17

Table 1. Continued

NDV strains	Genotype or subgroup	Accession number	Reference number
FIN-1-96	VII	AF091623	15
TR-8-97	VII	AF136785	14
AE-232-1-96	VII	AF109884	14
IT-1-00	VII	AF293350	13
IR-283-97	VII	AY135746	7
BG-109-84	VII	AF402133	10
ZA-360-95	VII	AF109876	8
ZW3422-95	VII	AF108877	8
DE-372-86	VII	AF525390	19
TW-95	VII	U62620	17
IT-112-84	VII	AF218127	13
IT-113-85	VII	AF218128	13

NDV strains of genotype VII and allowed us to determine that these strains were unique but most closely related to genotype VIIb.

Phylogenetic Relationships among NDV Isolates

Phylogenetic analysis of NDV strains isolated in Kazakhstan and study of their phylogenetic relationships with other NDV worldwide isolates was completed based on sequence analysis of the variable region of the F gene (47-421) (Figs. 2–3). Included in the analysis were NDV strains belonging to genotype groups I–VIII (see Table 1). The phylogenetic analysis demonstrated that the 10 NDV strains isolated in Kazakhstan and 65 representative NDV strains could be distributed in eight distinct clusters corresponding to the different genotypes. All 10 NDV strains isolated in Kazakhstan were assigned to genotype VII. The phylogenetic analyses have shown that Kazakhstan isolates and 39 representative strains of genotype VII which were taken from the NDV worldwide database could be distributed into 5 distinct clusters: VIIa, VIIb, VIIc, VIId, and VIIe and all 10 NDV strains isolated in Kazakhstan were assigned to subgroup VIIb. It was determined by analysis of this molecular data that the NDV strains isolated in Kazakhstan possess nucleotide substitutions unique to these isolates. The nucleotide substitutions characteristic for Kazakhstan NDV strains were found in positions 55 (G changed to T), 132 (G changed to A), 272 (T changed to C), 282 (C changed to T), and 303 (G changed to A). Based on nucleotide sequence analysis, a unique amino acid substitution (V^{19} to F^{19}) was found in all 10 NDV strains isolated in Kazakhstan.

Discussion

NDV strains responsible for sporadic NDV epizootics among poultry in European and Asian countries were tested using sequencing accompanied by phylogenetic analysis. It was demonstrated that pathogenic NDV strains that caused epizootics in Europe (Germany, Belgium, The Netherlands, Spain, and Italy, between 1992 and 1996), and in Asia (Japan, Taiwan, China, 1990) belonged to genotype VII [10–21]. Ten NDV strains isolated in Kazakhstan during 1998 examined according to their biological and molecular characteristics also belonged to genotype VII. The NDV strains isolated in Kazakhstan possess unique substitutions in nucleotide sequence characteristic only for strains isolated on the territory

Table 2. Biological and molecular characteristics of virulent NDV isolates recovered from NDV outbreak of chickens in Kazakhstan during the 1998 NDV

Isolate identification	Strain abbreviation	Host	ICPI	MDT	Fusion protein cleavage site (molecular pathotyping)
APMV-1/chicken/Almaty/22/98	KAZ-22-98	fowl	1.64	54.2	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/26/98	KAZ-26-98	fowl	1.05	85.6	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/AImaty/28/98	KAZ-28-98	fowl	1.68	51.4	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/30/98	KAZ-30-98	fowl	1.58	60	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/31/98	KAZ-31-98	fowl	1.61	54.9	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/34/98	KAZ-34-98	fowl	1.78	56	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/36/98	KAZ-36-98	fowl	1.58	59.2	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/38/98	KAZ-38-98	fowl	1.87	62.4	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/39/98	KAZ-39-98	fowl	1.42	70.4	^{112}RRQRR^{116} * F^{117}
APMV-1/chicken/Almaty/43/98	KAZ-43-98	fowl	1.56	58.8	^{112}RRQRR^{116} * F^{117}

Table 3. Deduced amino acid sequences of NDV F-gene fragment (variable region) provide the basis for differentiation of NDV genotypes

	Deduced amino acid sequences																	
NDV strains/genotypes	5 S	9 I	10 P	13 L	19 I	23 L	28 L	63 V	93 T	101 R	104 G	107 S	109 S	112 R	113 R	114 Q	115 K	121 I
VIIa	P	T				S				K			P				-/R	V
VIIb	P				V		S							-/K				V
KAZ-22-98	P				F		S							R				V
KAZ-26-98	P				F		S							R				V
KAZ-28-98	P				F		S							R				V
KAZ-30-98	P				F		S							R				V
KAZ-31-98	P				F		S							R				V
KAZ-43-98	P				F		S							R				V
KAZ-34-98	P				F		S							R				V
KAZ-36-98	P				F		S							R				V
KAZ-38-98	P				F		S							R				V
KAZ-39-98	P				F		S							R				V
VIIc	P			P						K				K				N
VIId	P						-/P			K								V
VIIe	P									K								V

Description of NDV genotype VII was obtained from published data [7–29].
Representative strains, deduced amino acid sequences and alignments were chosen accordingly MegAlign program (Windows 32, MegAlign 5.00) in the Lasergene package (DNASTAR Inc. Madison, WI 53717, USA).

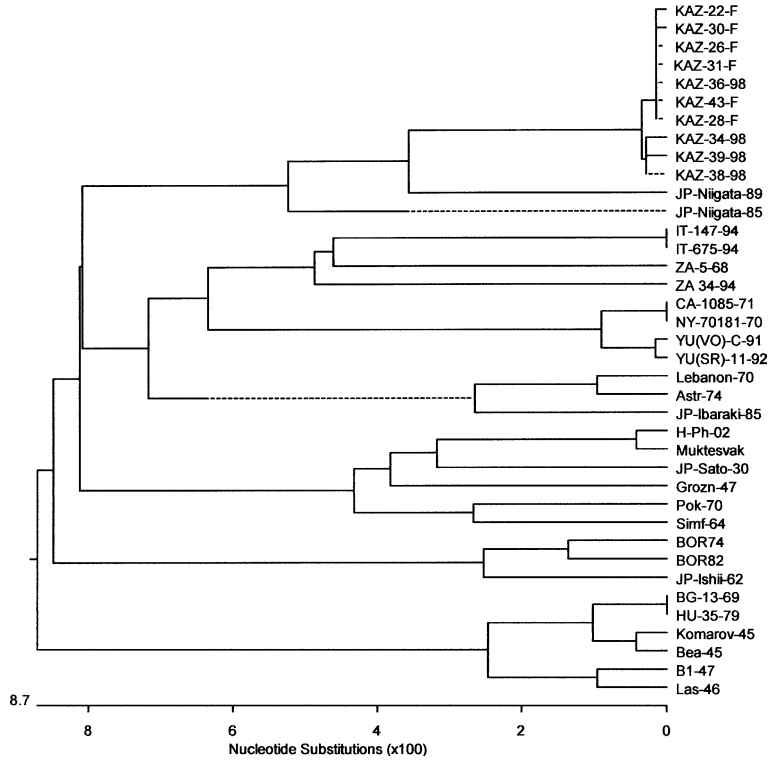

Fig. 2. Phylogenetic tree designed on the basis of nucleotide sequences of the F gene fragment (374 bp, 47-421) of NDV strains that belong to different genotypes.

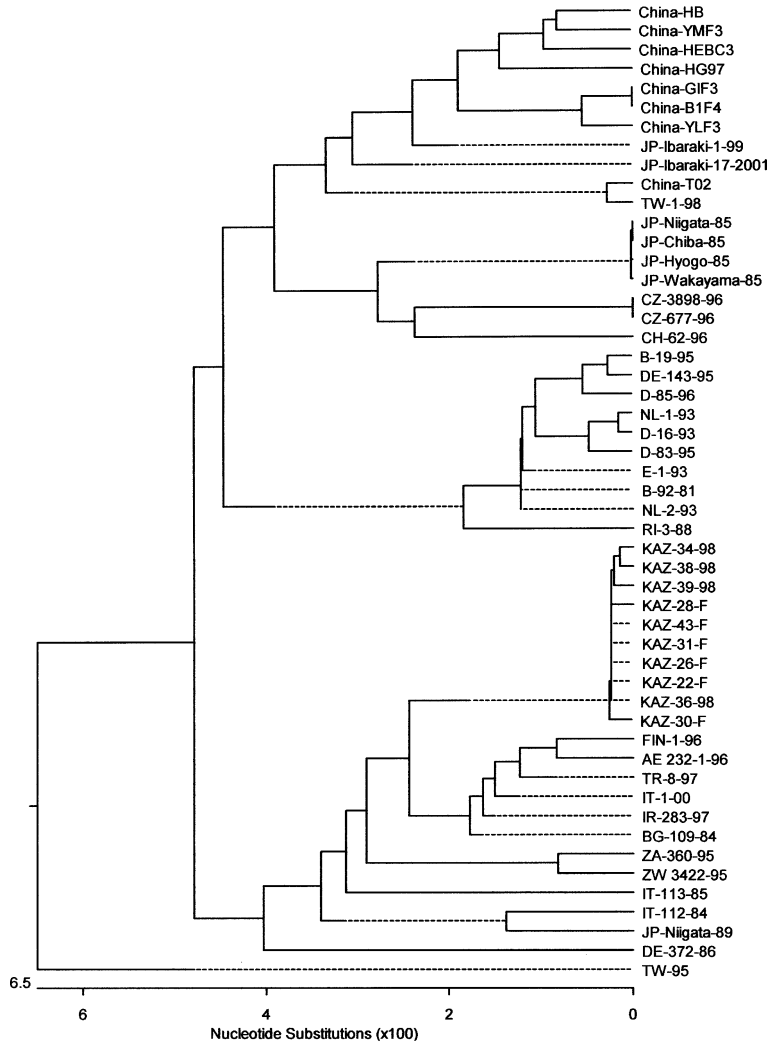

Fig. 3. Phylogenetic tree designed on the basis of nucleotide sequences of F gene fragment (374 bp, 47-421) of NDV strains that belong to genotype VII.

of Kazakhstan. Geographical-related substitutions were found in positions 55 (changes G to T), 132 (changes G to A), 272 (changes T to C), 282 (changes C to T), and 303 (changes G to A). Based on nucleotide sequence analysis, a unique amino acid substitution (V^{19} to F^{19}) was found in all 10 NDV strains isolated in Kazakhstan. On the basis of these data, 10 NDV strains isolated in Kazakhstan during 1998 were categorized as subgenotype VIIb.

One possibility for new nucleotide sequence substitutions in the genome of Kazakhstan NDV isolates may be related to selective pressures following intensive vaccination procedures among poultry farms in Kazakhstan. Another possibility may be introduction of new NDV strains from migrating birds during their direct contact with chickens in primarily open poultry farms in Kazakhstan. Further characterization of NDV strains circulating in Kazakhstan and other Central Asia countries is important for better control of NDV epizootics in the Central Asia region.

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